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Role of X-linked Inhibitor of Apoptosis in Breast Cancer

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14. ABSTRACT

XIAP is an exciting potential target for breast cancer therapeutics, but the specific functions of XIAP that contribute to breast cancer development or progression remain unknown. This project seeks to identify the properties of XIAP that play a crucial role in breast cancer, using mutated XIAP molecules that are deficient in specific cellular functions in both *in vitro* and *in vivo* models of breast cancer. In this phase of this project, cell lines deficient in XIAP have been evaluated to identify the role of XIAP in tumorigenesis, using models of anchorage-independent growth or tumor formation in immunocompromised mice. Understanding the effects of XIAP on tumor growth will a valuable guide in the development of therapeutics that specifically target tumor-related functions of XIAP and in the advancement of our understanding of breast cancer biology.

15. SUBJECT TERMS
Apoptosis, Breast Cancer, X-linked Inhibitor of Apoptosis

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Introduction

Pre-clinical evidence suggests that X-linked Inhibitor of Apoptosis (XIAP) is an exciting potential target for breast cancer therapeutics, which provides strong motivation for the role of XIAP in breast cancer to be fully elucidated. XIAP is involved in multiple cellular functions that have been implicated in breast cancer. This project seeks to identify the properties of XIAP that play a crucial role in breast cancer, by examining mutated XIAP molecules that are deficient in specific cellular functions in both in vitro and in vivo models of breast cancer.

Body

In the initial phase of this project, I evaluated a number of cancer cell lines to identify appropriate cells to establish models of breast cancer cell growth. I now have extensive experience with these models, and have chosen the MDA-MB-231 cell line we obtained from Marc Lippman as an excellent model of xenograft tumor growth in mice and anchorage-independent growth *in vitro*. In soft agar culture, suspended MDA-MB-231 cells grow into colonies over four to six weeks. In athymic nude mice, subcutaneous injection of MDA-MB-231 cells results in tumor development within four to six weeks. The tumor mass primarily consists of cells with advanced grade adenocarcinoma morphology and areas of necrosis on histologic examination.

To examine the effect of XIAP on cancer cell growth, I developed cell lines deficient in XIAP expression. The development of these cells was described in a previous report, and is briefly described here. Short hairpin RNA sequences (shRNA) were utilized to suppress XIAP expression using RNA interference (RNAi). These shRNA sequences were introduced into the cell lines using lentiviral vectors, resulting in integration into the genome and stable suppression of XIAP (Fig 1A). Stable, rather than transient, disruption of XIAP is necessary for the long duration of the *in vivo* and *in vitro* experiments proposed. The lentiviral vectors contain fluorescent markers, and cells were confirmed to express fluorescent proteins by FACS or by fluorescence microscopy (Fig 1B) and to demonstrate suppression of XIAP protein by Western blot (Fig 1C). In all, two cell lines were established with different XIAP shRNA sequences, in addition to two cell lines with different control shRNA sequences. These independently targeted cell lines will be particularly useful for identifying non-specific effects that could result from RNAi, lentivirus infection or off-target effects of a shRNA sequence. We desire to study specific mutations in XIAP that disrupt caspase-dependent and –independent functions (Lewis, 2004). Re-introduction of XIAP is accomplished by stable expression of lentiviral vectors, which allows the expression of mutated XIAP proteins to replace endogenous XIAP (Fig 2A). As above, XIAP expression was confirmed by Western blot (Fig 2B).

In new experiments since the previous annual report, XIAP-deficient cells have been examined for tumor formation *in vivo*. Preliminary experiments indicate that loss of XIAP correlates with a trend of

delayed tumor growth following subcutaneous injection in immunodeficient mice (Fig 3A). XIAP-deficient MDA-MB-231 cells are capable of forming large tumors, but at many injection sites growth is delayed 1-2 weeks. These experiments extend previously published results (LaCasse 2006, McManus 2004) demonstrating the importance of XIAP in tumor growth to breast cancer models using a long-term, non-pharmacological approach that allows further investigation of the effects of XIAP. However, within each group of tumors there is a large statistical variance in tumor size, and I have attempted to reduce the variance in tumor size by using rigorous techniques: XIAP-deficient and control cells were harvested from culture at identical cell density and injected on opposite flanks in the same mouse to eliminate mouse-to-mouse variation. Furthermore, mice were anesthetized during injection to ensure complete subcutaneous delivery of cells. To confirm that the appropriate cells are responsible for tumor formation, tumors were harvested and analyzed by Western blot for XIAP expression (Fig 3B). Additionally, tumors from mice injected with XIAP-deficient and control cells have been sub-cultured *in vitro*. Cells cultured from the tumor explants express appropriate fluorescent markers indicating they originated from the injected cells (Fig 3C). In the case of cells from XIAP shRNA expressing tumors, XIAP expression continues to be greatly diminished on Western blot (Fig 3D). Additional experiments will verify whether the effect of XIAP on tumor growth is reproducible comparing additional cell lines, including those reconstituted with XIAP.

In further tasks, I aimed to establish whether loss of XIAP in these cancer cell lines affects apoptosis, anchorage-independent cell growth and cell signaling. During initial experiments, I found that there is no difference in viability between XIAP-deficient cells and controls following treatment with apoptotic stimuli for up to 24 hours (Fig 4A). However, in further experiments the effect of XIAP on cell death can be seen at later times, 48 to 72 hours following apoptotic stimuli (Fig 4B). These results confirm that the XIAP-deficient cell lines used in the tumor studies are more sensitive to apoptotic stimuli, and the findings are currently in preparation for publication. As reported previously, XIAP-deficient cell lines did not demonstrate a difference in proliferation in normal cell culture conditions (Fig 5A) or a difference in the number of foci formed following growth in soft agar (Fig 5B). Given the new data on XIAP in apoptosis, these experiments may be combined to examine colony growth in soft agar for cells pre-treated with apoptotic stimuli, time permitting. In all, these studies confirm that XIAP does play a role in apoptosis in breast cancer cell lines, and experiments examining non-apoptotic roles of XIAP will be pursued further in the next phase of the project.

Key Research Accomplishments

- XIAP expression can be effectively suppressed using lentiviral introduction of shRNA in breast carcinoma cell lines.
- Loss of XIAP in MDA-MB-231 cells results in a trend of delayed growth in xenograft tumors. Suppression of XIAP persists in tumors in vivo and tumor explants ex vivo.
- Loss of XIAP does not alter breast cancer cell growth under normal culture conditions, but does increase sensitivity to apoptotic stimuli.

Reportable Outcomes

- Presenting author: University of Michigan Medical Scientist Training Program Retreat, Roscommon, MI, Aug 3-5, 2007. “The Role of X-linked Inhibitor of Apoptosis in Cancer Development and Progression,” Karolyn Oetjen, Clara Hwang, David Kosoff, Kirk Wojno, and Colin Duckett (poster).

- Presenting author: Keystone Symposium Cell Death and Cellular Senescence, Breckenridge, Colorado, Feb 7-12, 2008. “Innate immune responses to lipopolysaccharide require c-IAP1,” Karolyn Oetjen, Rebecca Csomos, John Wilkinson, Brian Rudd, Claudia Benjamim, Steven L. Kunkel , Nicholas Lukacs, Colin Duckett (poster).

- Contributing author: Keystone Symposium Cell Death and Cellular Senescence, Breckenridge, Colorado, Feb 7-12, 2008. “Analysis of XIAP in a murine model of human X-linked lymphoproliferative syndrome: susceptibility to g-herpesvirus infection,” Julie M. Rumble, Karolyn A. Oetjen, Casey W. Wright, Paul Stein, Pamela L. Schwartzberg, Beth B. Moore, Colin S. Duckett (poster).

Conclusion

XIAP has been identified as a potential target for breast cancer therapeutics, but the specific functions of XIAP that contribute to breast cancer development or progression remain unknown. The major tasks for this phase of this project involved evaluating breast cancer cell lines depleted in XIAP expression in tumor growth models *in vivo* and anchorage-independent growth models *in vitro*. These cell lines have been evaluated for sensitivity to apoptotic stimuli, and cell signaling in these cell lines is currently under investigation. In the next phase of this project, cell lines with rescued expression of XIAP or mutated XIAP will be examined. This knowledge will be valuable to guide the development of therapeutics that specifically target tumor-related functions of XIAP.

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Lewis J, Burstein E, Reffey SB, Bratton SB, Roberts AB and Duckett CS. Uncoupling of the signaling and caspase-inhibitory properties of X-linked inhibitor of apoptosis. J Biol Chem 279:9023-9. (2004)

McManus DC, Lefebvre CA, Cherton-Horvat G, St-Jean M, Kandimalla ER, Agrawal S, Morris SJ, Durkin JP, Lacasse EC. Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. Oncogene 23:8105-17. (2004)

Appendices

Figures 1-5 referenced in the text are appended.

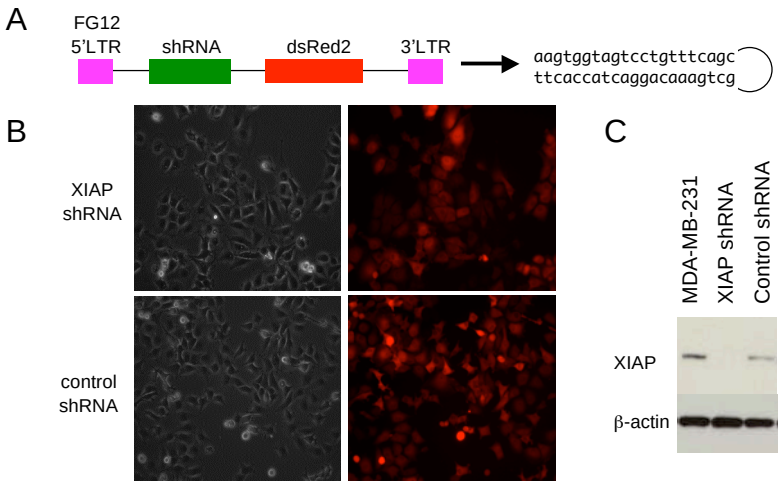

Figure 1. Stable suppression of XIAP in human breast carcinoma cells by RNAi. (A) A short hairpin sequence targeting XIAP (or control hairpin) was cloned into the bicistronic lentiviral vector FG12. Virus was packaged in 293T cells, and MDA-MB-231 cells were infected with viral supernatant. (B) Effective transduction of the lentivirus was confirmed by fluorescence microscopy. Light microscopic images are included for reference. (C) XIAP expression was evaluated by Western blot in parental MDA-MB-231 cells (left) and MDA-MB-231 cells transduced with XIAP shRNA (middle) or control shRNA (right). β-actin is included to control for protein loading (bottom).

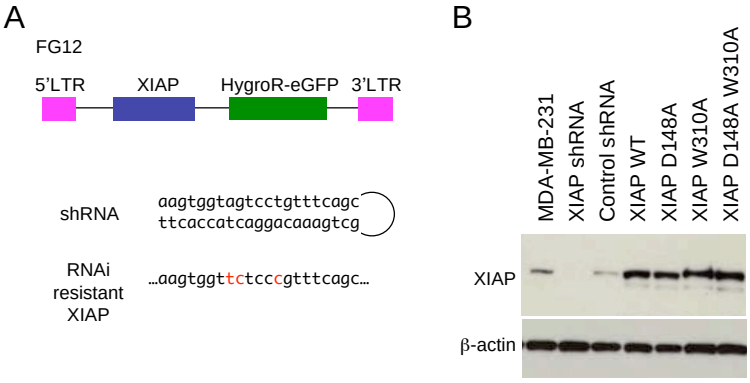

Figure 2. Re-expression of XIAP in shXIAP-expressing MDA-MB-231 cells. (A) Silent mutations were generated in the shRNA-targeted sequence of XIAP cDNA to create constructs resistant to RNAi-mediated suppression. Additional mutations at D148, W310 and H467A were also cloned, and the constructs were expressed using the FG12 lentiviral system. (B) Transduced MDA-MB-231 shXIAP cells were examined for XIAP expression by Western blot.

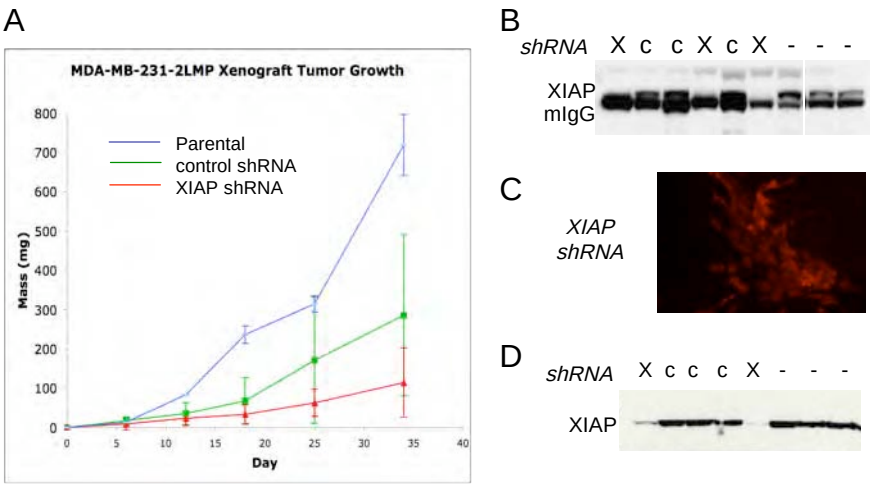

Figure 3. Xenograft tumor growth. (A) Athymic nude mice were subcutaneously injected with $3x10^6$ MDA-MB-231 cells in 100µL PBS bilaterally, and tumor growth was monitored by two perpendicular caliper measurements over 6 wks. Tumor mass was estimated as $a^2*b/2$, where a<b. (B) Lysates of tumor specimens were probed with XIAP mouse monoclonal antibody by Western blot to confirm persistent knock-down of XIAP. (C) Cells from tumor explants were sub-cultured ex vivo. Expression of dsRed2 marker in cultures from XIAP shRNA tumors was verified by fluorescent microscopy. (B, D) Abbreviations for samples: X=XIAP shRNA, c= control shRNA, - = parental MDA-MB-231 cells.

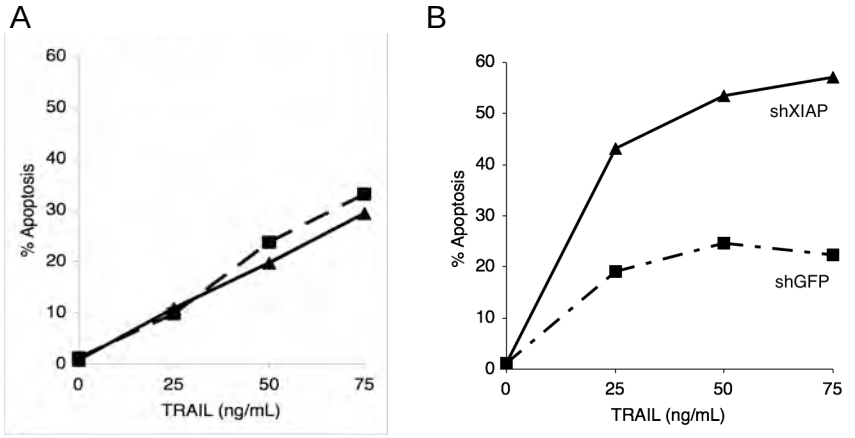

Figure 4. XIAP-dependent sensitivity to TRAIL. (A) MDA-MB-231 cells expressing shXIAP (solid) or control shRNA (dash) were treated with TRAIL for 2 hours and analyzed by flow cytometry for propidium iodide-positive cells immediately after treatment. (B) Cells were treated as before and analyzed 48 hours after treatment.

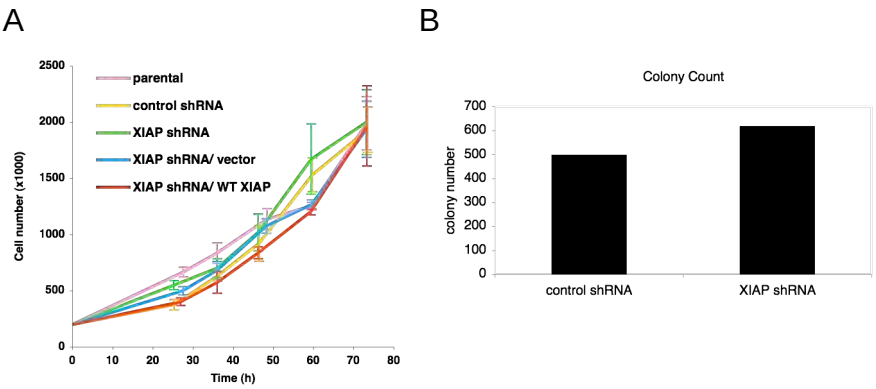

Figure 5. Loss of XIAP in breast carcinoma cells *in vitro*. (A) Identical numbers of MDA-MB-231 cells were plated in RPMI with full serum, and cell quantity was measured over three days. (B) MDA-MB-231 cells were plated in soft agar, and media was replenished as needed. Foci formation was monitored for 4-6 weeks by fluorescence microscopy.